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Interleukin 2-Bax: a novel prototype of human chimeric proteins for targeted therapy

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Abstract **During the past few years many chimeric proteins have been developed to target and kill cells expressing specific surface molecules. Generally, these molecules carry a bacterial or plant toxin that destroys the unwanted cells. The major obstacle in the clinical application of such chimeras is their immunogenicity and non-specific toxicity. We have developed a new generation of chimeric proteins, taking advantage of apoptosis-inducing proteins, such as the human Bax protein, as novel killing components. The first prototype chimeric protein, IL2-Bax, directed toward IL2R-expressing cells, was constructed, expressed in *Escherichia coli* and partially purified. IL2-Bax increased the population of apoptotic cells in a variety of target T cell lines, as well as in human fresh PHA-activated lymphocytes, in a dose-dependent manner and had no effect on cells lacking IL2R expression. The IL2-Bax chimera represents an innovative approach for constructing chimeric proteins comprising a molecule that binds a specific cell type and an apoptosis-inducing protein. Such new chimeric proteins could be used for targeted treatment of human diseases.**

Key words: Interleukin 2; Bax; Apoptosis; Targeted therapy; Chimeric protein

1. Introduction

The advent of the monoclonal antibody and recombinant DNA technologies has led to the discovery of numerous cell surface molecules associated with specific cell populations. Based on the differential expression patterns of these molecules, immunotoxins have been constructed to specifically target and destroy cells expressing such surface molecules, among them cancer cells and cells involved in disorders of the immune system. The targeting moiety of the immunotoxins consists of whole antibodies or antigen-binding domains, including the Fv portion of an antibody (single-chain immunotoxins) or growth factors and cytokines (chimeric proteins). These molecules are generally fused to various mutant forms of bacterial or plant toxin molecules to destroy the unwanted cells [1]. The major drawback in the clinical application of these molecules is the human immune response they elicit, mainly toward the toxin moiety. Bacterial toxins like *Pseudomonas* exotoxin A (PE) and diphtheria toxin are highly immunogenic and cannot be humanized by standard techniques.

In addition, each immunotoxin displays some degree of non-specific toxicity, and, at sufficiently high concentrations, damages normal cells that do not express the specific target antigen. This non-specific toxicity of immunotoxins/chimeric proteins is the dose-limiting factor in immunotoxin therapy. Thus, it has become clear that new approaches are needed to produce novel targeting molecules, mainly composed of human, non-immunogenic toxins.

We have developed a new generation of chimeric proteins, exploiting apoptosis-inducing proteins, such as the human Bax protein, as novel killing components instead of the troublesome bacterial or plant toxins. Bax is a pro-apoptotic member of the Bcl-2 protein family. Bax hetrodimerizes with Bcl-2 in vivo [2] but is also able to regulate apoptosis independently [3,4]. Induction of Bax expression is sufficient to induce apoptosis and does not require any additional death stimulus [5]. Bax forms channels in lipid membranes [6,7] and directly induces the release of cytochrome *c* from isolated mitochondria [8]. Recently, both Bax frame-shift mutations and loss-of-function mutations [9,10] were detected in hematopoietic malignancies and were found to be associated with resistance to apoptosis [9].

Moreover, elevations in Bax protein levels are induced in several clinically relevant settings where cell death occurs, including tumor cells during responses to chemotherapy and radiation [11,12], neurons following cerebral ischemia [13] and myocardiocytes following acute myocardial infarction [14].

To validate the use of pro-apoptotic proteins as the killing moiety in chimeric proteins, we chose a well known target, the interleukin 2 receptor (IL2R). IL-2-toxin chimeric proteins, such as IL2-*Pseudomonas* exotoxin (IL2-PE), successfully eliminate activated T cells, both in vitro and in vivo [15]. Another chimeric protein, DAB386-IL2, is now in clinical trials in CTCL patients [16,17]. In addition, the α chain of the IL2R was successfully targeted by the murine or the humanized anti-Tac antibody in cancer patients as well as in patients after transplantation [18,19].

In the present work we prove that the novel chimera, IL2-Bax, specifically targets IL2R-expressing cells and induces cell-specific apoptosis.

2. Materials and methods

2.1. Construction of a plasmid encoding the IL2-Bax protein; expression of the protein and partial purification

pHL906 [20], which carries the fusion gene IL2-PE40, was cut with *Hin*dIII and *Ppu*MI, removing the PE sequence, and the vector fragment was eluted.

The cDNA for human Baxα was obtained by RT-PCR, using RNA isolated from fresh human lymphocytes. Total RNA was isolated and was then reverse transcribed into first strand cDNA, using the reverse transcription system (Promega, USA) according to the manufacturer's instructions. The cDNA was diluted to a total volume of 1 ml with 10 mM Tris-HCl, pH 7.6, 1 mM EDTA and stored at 4°C until use. The Bax fragment was generated by PCR, using the above cDNA and

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a pair of synthetic oligonucleotide primers: 5'-CGGAATTCAAGCTTTGGACGGGTCCGGGGGA-3' (sense) and 5'-CGGAATTCAGGTCCTTCAGCCCATCTTCTTC-3' (antisense) covering the entire coding region. The reaction mixture was incubated in a DNA thermal cycler (MJ Research Inc., Watertown, MA, USA) for 33 cycles. Each cycle consisted of 1 min at 95°C, 1 min at 65°C and 2 min at 72°C. The Bax fragment, after digestion with *Ppu*MI and *Hin*dIII enzymes, was ligated to the vector fragment. The resulting plasmid pAY1 carries the Bax human sequence 3' to the IL2 sequence. The construct was confirmed by restriction endonuclease digestion and DNA sequence analysis.

The chimeric proteins were expressed in *Escherichia coli* strain BL21(λDE3). A pellet of expressing cells was suspended in 20 mM Tris-HCl pH 8.0, 1 mM EDTA containing 0.2 mg/ml lysozyme, sonicated (three 30-s bursts) and centrifuged at 35000×*g* for 30 min. The supernatant (soluble fraction) was removed and kept for analysis. The pellet was denatured in extraction buffer A (6 M guanidine-HCl, 0.1 M Tris-HCl, pH 8.6, 1 mM EDTA, 0.05 M NaCl, and 10 mM DTT) and stirred for 1 h at 4°C. The suspension was cleared by centrifugation at 35000×*g* for 15 min and the pellet discarded. The protein solution was diluted 1:100 in refolding buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.25 M NaCl and 5 mM dithiothreitol) and kept at 4°C for 48 h.

Two microgram (total protein) of the various fractions was run on a 12% SDS-PAGE gel.

2.2. Western blot analysis of cell fractions expressing IL2-Bax

Samples of the various protein fractions were Western blotted and analyzed with anti-Bax (Pharmingen, San Diego, CA, USA, 1:2500) and anti-IL2 (Endogen, Boston, MA, USA, 1:5000), using the ECL kit for detection.

2.3. FACS analysis

Cells (0.5×10^6/10 ml) were centrifuged at 450×*g* for 6 min, washed with cold PBS and resuspended in 300 μl of PBS. The cells were fixed with 5 ml methanol and left at −20°C for at least 1 h. The cells were then centrifuged at 800×*g* for 5 min, resuspended in 100 μl PBS and diluted to a final volume of 1 ml with PBS. Cells were incubated on ice for an additional 30 min, centrifuged at 800×*g* for 5 min and resuspended in 0.5 ml PBS. 10 μl RNase (50 μg/ml) and propidium iodide (PI, 5 μg/ml) were added to the cell samples which were then FACS analyzed for DNA content as a function of cell number.

All centrifugations were carried out at 4°C. All solutions used were chilled to 4°C before use.

For competition assays, 10 μg/ml rIL2 or anti-Tac (Genzyme, Cambridge, MA, USA) and IL2-Bax was added at a final concentration of 5 μg/ml for 16–18 h. Samples were analyzed with a FACScan (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA) using the LYSYS II program.

2.4. Preparation of fresh activated human lymphocytes

Human peripheral blood lymphocytes from healthy donors were isolated on a Ficoll-Isopaque gradient (1.077) and used immediately. Lymphocytes were cultured in 5% CO_2 in air in RPMI 1640 medium supplemented with 10% fetal calf serum, 200 mg/ml L-glutamate, 50 mg/ml penicillin, 50 mg/ml streptomycin, 50 mg/ml glutamine, 5×10^{-5} M β-mercaptoethanol and PHA (5 μg/ml) for 3 days. The activated cells were washed and resuspended in the presence of 10 U/ml rIL2 to maintain cell viability. Increasing concentrations of IL2-Bax were then added to the activated lymphocytes for 24 or 48 h and the cells were then stained with PI (5 μg/ml) and FACS analyzed.

2.5. TUNEL assay

We used the apoptosis detection system, fluorescein (Promega, USA) and the slides were screened with a Zeiss Axoplan microscope to visualize the fluorescein-12-dUTP labeled DNA. A color filter (filter set 23, with dual excitation at 485 and 546 nm) was used. Photographs were taken with ASA/400 color film (exposure time 15–60 s).

Cells (0.5×10^6/10 ml) were incubated overnight with 5 μg/ml of IL2-Bax.

2.6. Detection of DNA fragmentation

DNA was extracted from 2B4 cells exposed to dexamethasone (5×10^{-6} M) or IL2-Bax (5 μg/ml) for 24 or 48 h as described in [21] and run on 1.5% agarose for detection of DNA ladders.

3. Results

3.1. Construction and expression of the plasmid encoding IL2-Bax

We constructed a plasmid (pAY1) for the expression of the chimeric protein, under the control of a T7 promoter (Fig. 1A). The plasmid was transformed into *E. coli* strain BL-21 (λDE3) and the chimeric protein, termed IL2-Bax, was expressed. IL2-Bax, mainly found in the insoluble fraction of the expressing cells (Fig. 1B), reacted with both anti-Bax and anti-IL2 when tested by Western blot analysis (Fig. 1C), confirming the cloning and production of the in-frame full-length chimeric protein. The insoluble fraction of expressing cells was denatured and refolded, and this partially enriched protein preparation was used in the following experiments.

3.2. Effect of IL2-Bax on target cells

We evaluated the effect of IL2-Bax on HUT102 cells [22], human activated T cells expressing IL2R, by closely monitoring the viability of the cultured cells exposed to the new chimera. IL2-Bax efficiently inhibited cell growth and eventually led to cell death (Fig. 2A,B).

As a more direct test of IL2-Bax function, we measured the induction of apoptosis in the target cells by FACS analysis. IL2-Bax caused an increase in the population of apoptotic cells in HUT102 cells in a dose-dependent manner, as assessed by cell cycle staging of the cells with PI (Fig. 3). The highest response was observed at 5 μg/ml ($=1.4\times10^{-7}$ M of IL2-Bax) of the chimeric protein.

IL2-Bax also induced apoptosis in various other IL2R-positive T and B cell lines (Table 1) that express various levels and species of the IL2R chains [23,24]. The chimeric protein induced apoptosis in the murine 2B4 T cell line as well.

IL2-Bax induced apoptosis not only in established cell lines but also in fresh human PHA-activated lymphocytes obtained from control donors (Fig. 4). This effect could be blocked by anti-Tac (Fig. 4).

3.3. Specificity of IL2-Bax response

IL2-Bax displayed specific activity, as it was inhibited by recombinant IL2 (Fig. 3) or anti-Tac, a monoclonal antibody against the IL2R-α chain, that blocks the binding of IL2 to its high affinity receptor (Fig. 3).

As a control we used the chimeric protein IL2-PE, which targets the same cells but carries the classical bacterial toxin PE as the killing moiety. IL2-PE did not cause any measurable apoptotic effect in the target cells, although it caused inhibition of protein synthesis within the cells (results not shown).

Table 1
Percent of apoptotic cells in IL2R-positive cell lines exposed to IL2-Bax

Cell line	IL2-Bax (5 μg/ml) % apoptotic cells[a]
HUT102	43.2
MT-1	29.8
ARH-77	25.8
2B4	18.3

[a]Results are expressed as the mean of 3–4 experiments performed for each cell line. Experiments were repeated with variations of no more than ±3%.

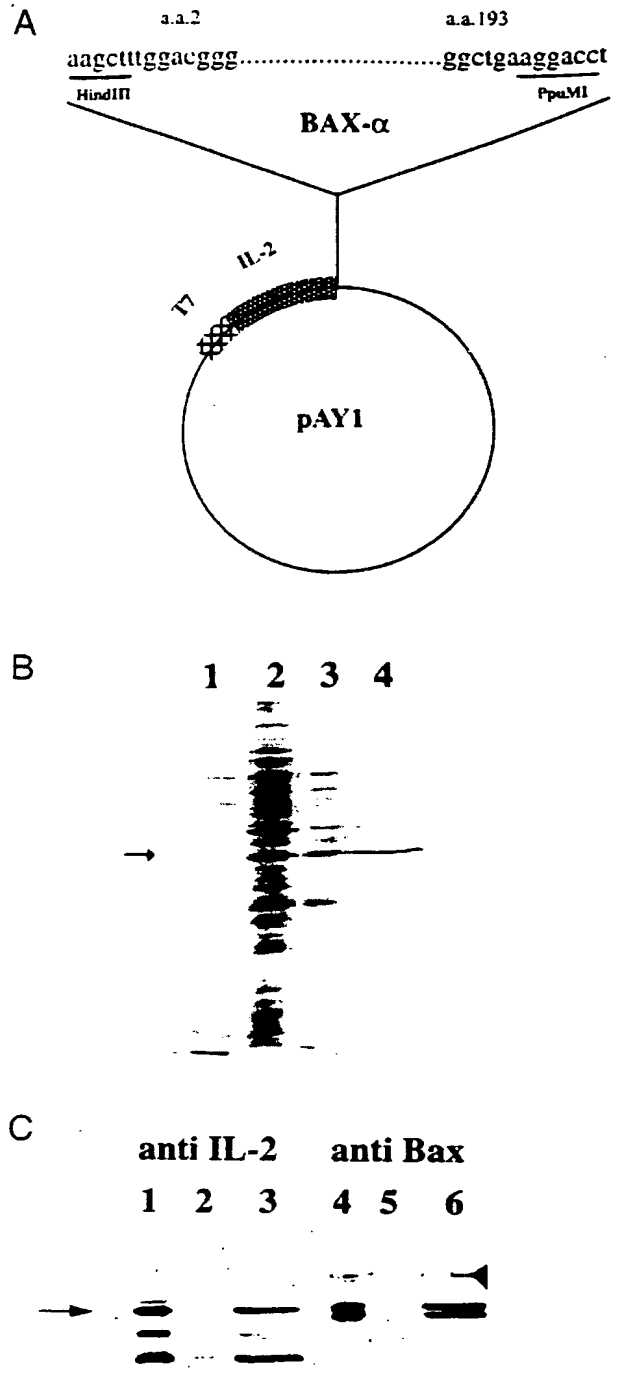

Fig. 1. A: Construction of the plasmid pAY1 that encodes the IL2-Bax. The numbers represent the corresponding amino acids. B: SDS-PAGE analysis of cell fractions containing the IL2-Bax chimeric protein. 2 μg (total protein) of the various fractions was run on a 12% SDS-PAGE gel. Lane 1: marker; lane 2: whole cell extract of cells expressing IL2-Bax; lane 3: soluble fraction of the expressing cells; lane 4: insoluble fraction of the expressing cells. Arrow indicates the IL2-Bax protein. C: Western blot analysis of cell fractions containing IL2-Bax. Lanes 1 and 4: whole cell extract of cells expressing IL2-Bax; lanes 2 and 5: soluble fraction of the expressing cells; lanes 3 and 6: insoluble fraction of the expressing cells. Lanes 1–3, using anti-IL2 antibodies. Lanes 4–6, using anti-Bax antibodies. Arrow indicates the IL2-Bax protein. For experimental details see Section 2.

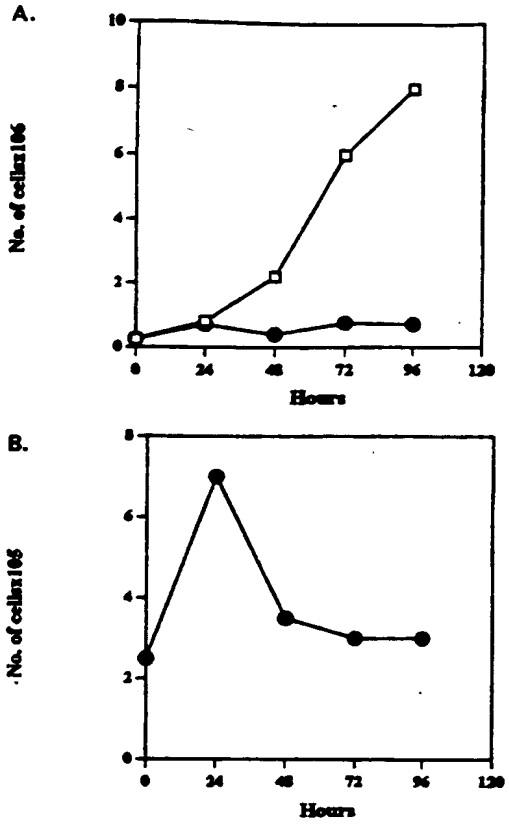

Fig. 2. Effect of IL2-Bax on HUT102 cell growth. A: Total number of cells in cultures in the presence (–●–) or absence (–□–) of IL2-Bax (2.5 μg/ml). B: Number of living cells in culture exposed to IL2-Bax (2.5 μg/ml). Cells were stained with trypan blue (0.4%).

IL2-Bax did not have any effect on cells lacking IL2R expression, such as CEM (a human T cell line), Km3 (a human non-T, non-B stem cell line), Nalm 6 (a human pre-B cell line), or on cell lines of non-hematopoietic origin such as Colo205 (a human colon adenocarcinoma cell line) and 293 cells (a human renal adenocarcinoma cell line) (Table 2).

We tested also other control chimeric proteins composed of various irrelevant targeting moieties. None of the control proteins induced apoptosis in HUT102 target cells (results not shown).

3.4. Detection of apoptosis caused by IL2-Bax

Induction of apoptosis by IL2-Bax was also confirmed using the annexin-V assay on HUT102 cells for early detection of apoptosis. The apoptotic effect was already evident at 6 h post exposure to IL2-Bax, and peaked at 24 h (results not shown).

As further confirmation of apoptosis we obtained TUNEL-positive HUT102 cells after 24 h exposure to IL2-Bax (Fig. 5A). Similar results were observed with 2B4 cells (results not shown). Nuclear changes, i.e. DNA fragmentation, induced by IL2-Bax can be seen at a later stage (48 h) in the 2B4 cells (Fig. 5B). Dexamethasone, a known inducer of apoptosis in various cells, was used in all experiments as a positive control.

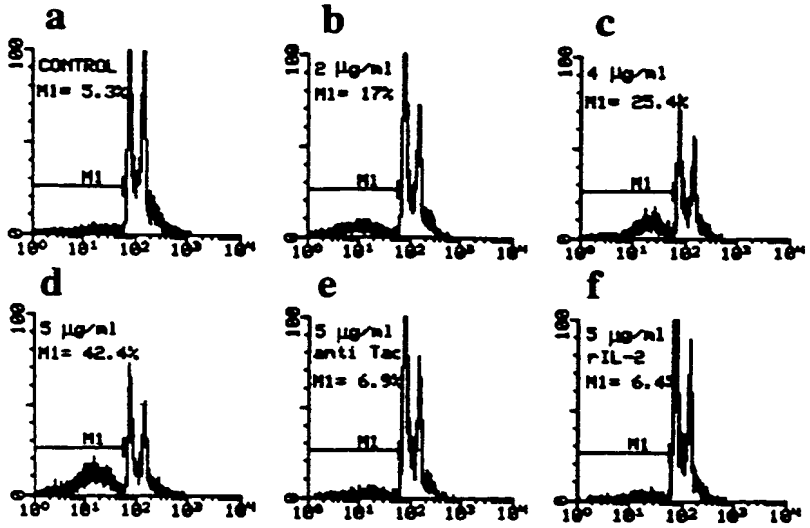

Fig. 3. Induction of apoptosis in HUT102 cells by IL2-Bax: FACS analysis. a–d: dose response; e: competition by anti-Tac; f: competition by rIL2. Samples were FACS analyzed for DNA content (*x*-axis) as a function of cell number (*y*-axis) as described in Section 2.

4. Discussion

The development of specific targeted agents remains a major goal in the treatment of human diseases. The hallmark of an effective therapeutic modality is its specificity, the ability to interact with the target organ and its lack of undesirable effects in the human recipient.

We report here our novel approach for developing chimeric proteins that can be used for targeted therapy in human diseases. The first prototype molecule, IL2-Bax, specifically targets IL2R-expressing cells and induces cell-specific apoptosis.

IL2-Bax induced apoptosis in the target cells in a dose-dependent manner (Fig. 3). The strongest response to IL2-Bax was observed at 1.4×10^{-7} M (Fig. 3) of the chimeric protein. These concentrations of Bax are within the physiological range as it has been previously reported that basal concentrations of Bax protein in a variety of human tumor cell lines can be as high as $\sim 0.1 \times 10^{-6}$ M [8], and Bax protein levels can rise in some tumor cells by over 10-fold following exposure to chemotherapeutic drugs or γ-radiation [11].

The novel chimeric protein, carrying the human Bax protein, induced apoptosis also in the mouse 2B4 T cell line, in accordance with a recent report demonstrating that a specific domain of Bax (aa 112–192) was responsible for mammalian apoptosis in a murine promyeloid cell line, FDC-P1, as well as for bacterial death [25].

Our results show that targeting of the Bax protein via the IL2R in the form of a chimeric protein is sufficient to induce apoptosis in the target cells in the absence of any additional death stimulus. To elucidate the mechanism by which IL2-Bax induces apoptosis, we used confocal microscopy to follow the path of IL2-Bax within the treated cells. Preliminary results revealed that following 24 h exposure to IL2-Bax, when apoptosis is already evident, the chimera colocalizes with the mitochondria, suggesting that IL2-Bax may play a similar role to the natural endogenous Bax protein (R. Aqeilan and H. Lorberboum-Galski, manuscript in preparation).

Although proteins such as Bax are intracellular proteins, they are of human origin and, as such, they are expected to display reduced immunogenicity in human recipients. Furthermore, killing target cells via the apoptotic pathway, we minimize any tissue damage or systemic response. The apoptotic cells shrink and condense, while the organelles and plasma membranes retain their integrity, then the dead cells are rapidly phagocytosed by neighboring cells or macrophages, disappearing before there is any leakage of their content.

IL2-Bax is now being developed for treatment of graft re-

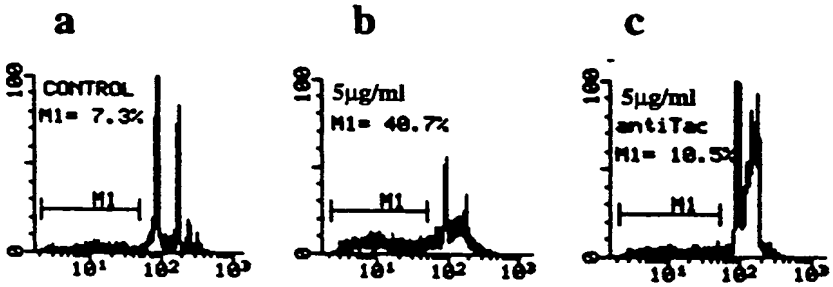

Fig. 4. Induction of apoptosis in human activated fresh lymphocytes by IL2-Bax. Human peripheral blood lymphocytes from healthy donors were separated, activated with PHA and measured for their response to IL2-Bax. Increasing concentrations of IL2-Bax were added to the activated lymphocytes for 24 and 48 h. Cells were then stained with PI (5 µg/ml) and FACS analyzed as described in Section 2 and Fig. 3.

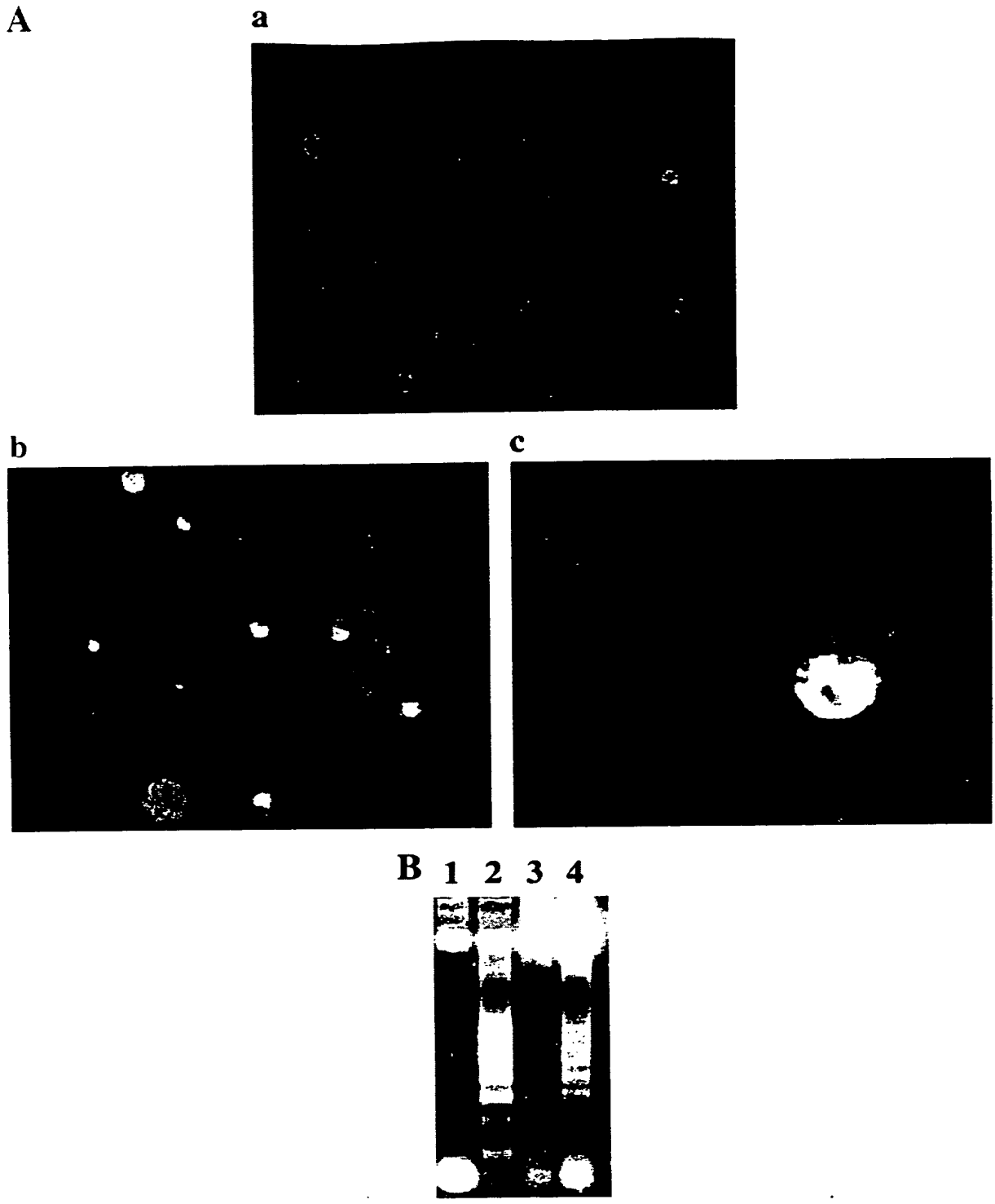

Fig. 5. A: TUNEL-positive HUT102 cells after exposure to IL2-Bax. a: control cells (×200); b: cells exposed to IL2-Bax (×200); c: single cell from b, magnified × 1000. Normal cells stain red, apoptotic cells stained yellow-red. B: DNA fragmentation in 2B4 cells exposed to IL2-Bax. For detection of DNA fragmentation, DNA was extracted from 2B4 cells exposed to dexamethasone (5×10^{-6} M) or IL2-Bax (5 μg/ml) for 24 or 48 h. Lane 1: DNA extracted from control 2B4 cells, not exposed to any inducer (48 h.); lane 2: DNA extracted from 2B4 cells exposed to dexamethasone (5×10^{-6} M) for 48 h; lane 3: DNA extracted from 2B4 cells exposed to IL2-Bax (3 μg/ml) for 48 h; lane 4: DNA extracted from 2B4 cells exposed to IL2-Bax (5 μg/ml) for 48 h. In both assays 0.5×10^{6}/10 ml cells were incubated with IL2-Bax.

Table 2
Effect of IL2-Bax on IL2R-negative cell lines

Cell line	Cell type	Percent of apoptotic cells[a]	
		Control	IL2-Bax (5 μg/ml)
CEM	T cell (IL2R−)	1.2	2.2
Km3	non-B, non-T stem cells	2.6	4.3
Nalm 6	pre-B cells	4.2	5.8
Colo205	colon-adenocarcinoma	7.2	9.8
293	renal adenocarcinoma	3.8	5.3
HUT102	T cells (IL2R+)	9.0	46.7

[a]Results are expressed as means of 3–4 experiments performed for each cell line. Experiments were repeated with variations of no more than ±3%.

jection, certain autoimmune diseases, infectious diseases and several malignancies.

IL2-Bax represents a novel approach for constructing chimeric proteins by fusing a targeting molecule, that binds to a specific cell type, to a wide range of apoptosis-inducing proteins. These novel chimeras are expected to selectively eliminate specific cell types both in vitro and in vivo.

Reagents such as IL2-Bax open up new prospects for the specific targeted therapy of human diseases.

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